AN ABSTRACT OF THE THESIS OF

Patricia A. Ormonde for the degree of Master of Science presented on April 14, 1995.

Title: Characterization of the Matrix Proteins of the Fish Rhabdovirus, Infectious Hematopoietic Necrosis Virus.

Redacted for Privacy

Abstract approved: _____

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Infectious hematopoietic necrosis virus (IHNV) is an important fish pathogen enzootic in salmon and trout populations of the Pacific Northwestern United States. Occasional epizootics in fish hatcheries can result in devastating losses of fish stocks. The complete nucleotide sequence of IHNV has not yet been determined. This knowledge is the first step towards understanding the roles viral proteins play in IHNV infection, and is necessary for determining the relatedness of IHNV to other rhabdoviruses. The glycoprotein, nucleocapsid and non-virion genes of IHNV have been described previously; however, at the initiation of this study, very little was known about the matrix protein genes. Rhabdoviral matrix proteins have been found to be important in viral transcription and virion assembly. This thesis describes the preliminary characterization of the M1 and M2 matrix proteins of IHNV. In addition, the trout humoral immune response to M1 and M2 proteins expressed from plasmid DNA injected into the fish was investigated. This work may prove useful in designing future vaccines against IHN.

The sequences of M1 phosphoprotein and M2 matrix protein genes of IHNV were determined from both genomic and mRNA clones. Analysis of the sequences indicated that the predicted open reading frame of M1 gene encoded a 230 amino acid protein with a estimated molecular weight of 25.6 kDa. Further analysis revealed a

second open reading frame encoding a 42 amino acid protein with a calculated molecular weight of 4.8 kDa. This second protein is highly basic and arginine rich, reminiscent of the small proteins encoded within the phosphoprotein gene of vesicular stomatitis virus (VSV) and rabies virus (RV). The putative M2 gene open reading frame encoded a 195 amino acid protein with a calculated weight of 22 kDa. In addition, the intergenic regions of the fish rhabdoviruses were compared. From the alingments of the intergenic sequence, the probable consensus transcriptional initiation signal for IHNV mRNA was determined. The amino acid sequences of the IHNV matrix proteins share sequence homology with that of the hirame rhabdovirus (HRV), viral hemorrhagic septicemia virus (VHSV) and no other rhabdoviruses for which there are available sequence data.

Since previous studies indicated that the M1 phosphoprotein and M2 matrix proteins could act as immunological adjuvants, plasmids were constructed for the expression of M1 or M2. The genetic immunization was carried out by direct injection of the plasmid DNA into rainbow trout. The specificity of the antibody response to the matrix proteins was determined by western blotting and serum antibody levels were monitored by enzyme-linked immunosorbant assays.

CHARACTERIZATION OF THE MATRIX PROTEINS OF THE FISH RHABDOVIRUS, INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS

by

Patricia A. Ormonde

A THESIS

submitted to

Oregon State University

in partial fulfillment
of the requirements for the
degree of

Master of Science

Completed April 14, 1995

Commencement June, 1995

Master of Science thesis of Patricia A. Ormonde presented on April 14, 1995

APPROVED:

Redacted for Privacy

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CHAPTER 1
INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is an important fish pathogen enzootic in salmon and trout populations of the Pacific Northwestern United States. Occasional epizootics in fish hatcheries can result in devastating losses of fish stocks. This thesis describes the characterization of the matrix proteins of IHNV.

Sequence analysis of the matrix proteins of IHNV

The complete nucleotide sequence of IHNV has not yet been determined. This knowledge is the first step towards understanding the roles viral proteins play in IHNV infection, and is necessary for determining the relatedness of IHNV to other rhabdoviruses. The glycoprotein, nucleocapsid and non-virion genes of IHNV have been described previously; however, at the initiation of this study, very little was known about the matrix protein genes. Rhabdoviral matrix proteins have been found to be important in viral transcription and virion assembly. The third chapter of this thesis describes the preliminary characterization of the M1 and M2 matrix proteins of IHNV.

The sequences of M1 phosphoprotein and M2 matrix protein genes of IHNV were determined from both genomic and mRNA clones. Analysis of the sequences indicated that the predicted open reading frame of M1 gene encoded a 230 amino acid protein with a estimated molecular weight of 25.6 kDa. Further analysis revealed a second open reading frame encoding a 42 amino acid protein with a calculated molecular weight of 4.8 kDa. This second protein is highly basic and arginine rich, reminiscent of the small proteins encoded within the phosphoprotein gene of vesicular

stomatitis virus (VSV) and rabies virus (RV). The putative M2 gene open reading frame encoded a 195 amino acid protein with a calculated weight of 22 kDa. In addition, the intergenic regions of the fish rhabdoviruses were compared. From the alignments of the intergenic sequence, the probable consensus transcriptional initiation signal for IHNV mRNA was determined. The amino acid sequences of the IHNV matrix proteins share sequence homology with that of the hirame rhabdovirus (HRV), viral hemorrhagic septicemia virus (VHSV) and no other rhabdoviruses for which there are available sequence data.

Humoral response in rainbow trout following DNA injection

Since previous studies indicated that the M1 phosphoprotein and M2 matrix proteins could act as immunological adjuvants, plasmids were constructed for the expression of M1 or M2. The fourth chapter of this thesis describes the genetic immunization by direct injection of the plasmid DNA into rainbow trout. The specificity of the antibody response to the matrix proteins was determined by immunoblotting and serum antibody levels were monitored by enzyme-linked immunosorbant assays. This work may prove useful in designing future vaccines against IHN.

CHAPTER 2
LITERATURE REVIEW

Infectious Hematopoietic Necrosis Virus

The *Rhabdoviridae* family is a large, diverse group of single-stranded RNA viruses. Over 150 rhabdoviruses have been described, approximately half of them in animals and the other half in plants (Peters, 1991). Infectious hematopoietic necrosis virus (IHNV), the causative agent of infectious hematopoietic necrosis disease in salmon and trout, is one of many rhabdoviruses that infect fish.

Distribution and host range of IHNV

Infectious hematopoietic necrosis virus infects trout and salmon in the Pacific Northwest of the United States and elsewhere. IHNV causes serious disease outbreaks in hatchery-reared trout and salmon and has been spread to other parts of the world by the movement of infected eggs and fry (Hill, 1992). The virus has been isolated from rainbow trout (*Oncorhynchus mykiss*) in France (Laurencin 1987), Taiwan (Chen et al. 1985), Italy (Bova et al. 1987) and more recently in Belgium (Hill 1992) and Korea (Park et al. 1993). In Japan, IHNV has become a serious pathogen with yearly outbreaks in hatcheries (Fukuda et al. 1992). IHNV is known to infect chinook salmon (*O. tshawytscha)*, sockeye salmon (*O. nerka*), rainbow trout (*O. mykiss*), cutthroat trout (*O. clarki*), Atlantic salmon (*Salmo salar*) and kokanee salmon or land-locked sockeye salmon (Pilcher and Fryer 1980; Wolf 1988). The disease is most severe in hatchery-reared fry where losses can sometimes approach 100%. Near yearling and yearling fish are also susceptible, but in older fish the histopathologic changes may be less severe and the mortality rates lower (Wolf 1988). Coho salmon (*O. kisutch*) appear to be

refractory to IHN disease and, although virus has been isolated from adult fish, no natural outbreaks have been reported (La Patra et al. 1989).

IHNV has been classified into 5 electropherotypes based upon the molecular weight of viral proteins following electrophoresis (Hsu et al. 1986). Members of the Round Butte, electropherotype 1 and Rangen, electropherotype 2 cause the majority of outbreaks in the Pacific Northwest of the United States.

IHNV causes a severe, acute, systemic disease which is accompanied by a rapid onset of mortality. External signs of disease include distention of the abdomen, hemorrhaging at the base of the fins, exophthalmia and behavioral changes (Pilcher and Fryer 1978 and Winton et al 1983). In surviving sockeye salmon, deformities such as scoliosis and a retracted head have been observed; these same deformities have not been reported in chinook salmon or rainbow trout (for a review, see Wolf 1988). Internally the fish appear anemic, due to blood loss. The digestive tract is devoid of food and the stomach and intestine contain mucus-like fluid; the body cavity may contain a serum-colored ascitic fluid. Additional signs include an unusually pale liver, kidney and spleen.

The primary targets of the disease are the renal and hematopoietic tissue of thekidney, pancreas, gastrointestinal tract and adrenal cortex. The most severe histopathologic changes occur in the hematopoietic tissue of the kidney and spleen, where the tissues are degenerative and necrotic. In the kidney these changes are accompanied by an increase in macrophages and a decrease in nondifferentiated blast cells. As the disease progresses, focal areas of cells show nuclear abnormalities, such as thickening of the nucleus, polymorphisms and margination of chromatin.

Morphology

IHNV are typical rhabdoviruses with bacilliform or bullet-shaped morphology. The particles have an average length of 160 nm and a core diameter of 60-70 nm

(Amend and Chambers 1970). The virion is composed a tightly coiled nucleocapsid surrounded by an envelope with protruding glycoprotein spikes. The matrix protein is generally thought to be positioned between the nucleocapsid coil and the membrane (Pal et al. 1987). Recently it has been suggested that the matrix protein of the rhabdovirus, vesicular stomatitis virus (VSV) forms a scaffold around which the nucleocapsid is wrapped (Gaudin et al. 1995).

Classification

The *Rhabdoviridae* family is divided into three genera and a plant rhabdovirus group (Table 1.1). VSV is the prototypic member of the *Vesiculovirus* and the rabies virus (RV) is the prototypic *Lyssavirus*. The *Lyssaviruses* that infect fish include : infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV) and the hirame rhabdovirus (HRV). The fish *Vesiculoviruses* include the pike fry rhabdovirus (PFR), spring viremia of carp (SVCV), Eel virus European X (EVEX) and Eel virus of America (EVA). This classification is based upon the gene order, sero-neutralization, monoclonal antibody reactivity studies and viral protein characterization (Tordo 1992). IHNV, VHSV and HRV exhibit a similar electrophoretic protein pattern and share antigenic determinants by Western blot analysis (Nishizawa et al. 1991). Although the fish rhabdoviruses share the same gene order as the *Lyssaviruses,* they do not exhibit sero-cross reactivity with the rabies virus (Peters, 1991).

Nucleic acid and proteins of IHNV

IHNV has been placed in the *Lyssavirus* genus of the *Rhabdoviridae* on the basis of the electrophoretic migration of the virion proteins (Coslett et al 1980). The viral genome is an unsegmented, single-stranded RNA of negative polarity (McCain et

al. 1974; Hill 1975). In 1985, Kurath and Leong reported the molecular weight for both genomic RNA (10.9 Kb) and viral mRNAs, and identified the corresponding proteins.

Table 2.1 Classification scheme of the *Rhabdoviridae* family

GENUS	SPECIES
Vesiculovirus	Vesicular stomatitis virus (VSV) Spring viremia of carp virus (SVCV) Pike fry rhabdovirus (PFR) Eel virus of America (EVA) Eel virus European X (EVEX)
Lyssavirus	Infectious hematopoietic necrosis virus (IHNV) Rabies virus (RV) Hirame rhabdovirus (HRV) Viral hemorrhagic septicemia virus (VHSV)
Bovine Ephemeral	Bovine Ephemeral virus (BEV)
Plant Rhabdovirus group	Sonchus yellow net virus (SYNV)

The IHNV genome encodes five virion associated proteins : L, polymerase; G, glycoprotein; N, nucleoprotein; M1, phosphoprotein; and M2, matrix protein. In addition, IHNV has been shown to encode a nonvirion-associated protein (NV) which has been found only in infected cell lysates (Kurath, 1985). The gene order of IHNV is (3') N - M1 - M2 - G - NV - L (5') as determined by R-loop electron micrographic analysis (Kurath et al. 1985). This is identical to VSV with its (3') N- P - M2 -G - L (5') gene order (Ball and White, 1976) and RV with its (3') N - M1 - M2 - G - L (5') gene order (Flamand and Delagneau 1978) with the exception of the NV in IHNV. During replication, the N is the first viral protein detected, appearing 2-3 hours after infection. This is followed by the synthesis M1 and M2 at 6-7 hours and the glycoprotein at 9-10 hours. Virus production begins approximately 12-14 hours after

infection (Leong et al. 1983). The N and M1 proteins of IHNV are the only proteins which are know to be phosphorylated (McAllister and Wagner 1975).

Sequence analysis of IHNV

The nucleotide sequence for the glycoprotein (G) gene (Koener et al. 1987), nucleocapsid (N) gene (Gilmore and Leong 1988) and the non-virion (NV) gene (Chiou, submitted) of IHNV have been determined. This information was important for the characterization of these viral proteins and enabled comparisons to be made with other rhabdoviruses.

The IHNV G is associated with the lipid bilayer and forms spike-like projections on the virion surface visible in electron microscopy (McAllister and Wagner 1975). The G of IHNV is approximately the same size as in VSV and rabies virus and shares many features characteristic of rhabdoviral glycoproteins such as a signal sequence, transmembrane domain and glycosylation sites (Koener et al. 1987). Comparison of the amino acid homology among VSV, RV and IHNV G proteins revealed a similar evolutionary distance among IHNV and VSV, and IHNV and RV (Koener et al. 1987).

Sequence analysis of the nucleocapsid protein showed no significant homology at the nucleotide level among the N genes of IHNV and VSV, rabies virus, or sonchus yellow net virus (SYNV), a plant rhabdovirus. However, amino acid comparisons indicated that there were amino acid sequences which were conserved among these viruses (Gilmore and Leong 1988). Now that the sequence information is available for SVCV and VHSV it would be of value to reexamine the relatedness of both of N and G among the fish rhabdoviruses.

Rhabdovirus transcription and replication

Rhabdovirus transcription and replication has been studied most extensively using the VSV as a model (for a review, see Banerjee and Barik 1992). It is thought that the same mechanisms for transcription and translation are used by IHNV. The negative-stranded RNA genome of rhabdoviruses serves as a template for both transcription of viral mRNAs and replication of the genome. The RNA-dependent RNA polymerase, composed of the large L protein and the phosphoprotein P, transcribes the RNA nucleocapsid protein (RNP) template to generate a leader RNA and 5' capped, 3' polyadenylated mRNAs (Banerjee, 1992). Inhibition of host cell transcription is dependent upon viral transcription (Weck and Wagner 1978) and was attributed to the leader RNA, but new evidence suggests that the M2 matrix protein is also involved (Black and Lyles 1992).

Before viral replication can occur, the negative sense genome must first be replicated into positive sense, full length genomic RNA (for review, see Banerjee 1987). Concentrations of nucleocapsid protein (N) regulate the switch from transcription to replication. During replication N binds to the 3' leader RNA and acts as an anti-terminator, resulting in full length, positive sense RNA with associated N protein (RNP). The positive RNP complex then serves as the template for the synthesis of full length, negative-stranded genomes.

Immune response following infection

The IHNV glycoprotein is the only viral protein capable of eliciting neutralizing antibodies (Engelking and Leong 1989) which were shown to provide protective immunity against five IHNV electropherotypes (Engelking and Leong 1989). This is also the case for VSV and RV, where virus-neutralizing antibodies in mammalian hosts are directed solely against the glycoprotein (Kelley et al. 1972; Cox et al. 1977; Wiktor

et al., 1984). However, Dietzschold et al. (1987) showed that protective immunity against rabies could be achieved by immunization with rabies virus ribonucleoprotein. These findings suggested that cytotoxic T-cell responses may also be important in protective immunity. Other studies investigating the B and T cell responses in mice reported a strong cytotoxic T-cell specific response against the phosphoprotein of RV(Larson et al. 1992). However, this response was unable to protect mice against challenge with RV. Unfortunately, at this time, T-cell specific responses cannot easily be monitored in fish; therefore, it is unknown whether cell-mediated responses contribute to protection against an IHN infection.

Rhabdovirus Matrix Proteins

The rhabdovirus matrix proteins include (M1) and (M2) of the *Lyssaviruses* and their analogs, the phosphoprotein (P) and (M) of the *Vesiculoviruses*. These protein have been described most thoroughly in using VSV has as a model system.

The phosphoprotein

The VSV phosphoprotein (P), originally called NS for nonstructural protein, is associated with the viral polymerase and is essential for viral transcription and replication (for review, see Banerjee and Barik 1992). The phosphoprotein is highly phosphorylated and acidic, with aspartic acid and glutamic acid residues making up 32% of the first 100 amino acids. Both of these characteristics are important to its function as a transcriptional activator. The phosphoprotein of VSV has been shown to consist of 3 distinct functional domains (Chattopadhyay and Banerjee 1988). Domain I is located towards the NH_2 terminus and contains clusters of negatively charged amino acids as well as constitutively phosphorylated residues. This region can be functionally replaced with β–tubulin, suggesting that the acidic charge and phosphorylation status

are important in maintaining function (Chattopadhyay and Banerjee 1988). The P protein is phosphorylated in a series of steps by cellular and viral kinases, both of which are required for transcriptional activation (Barik and Banerjee 1992). The identities of all of the kinases have not yet been determined; however, studies have shown that L is a virally-associated kinase and the cellular kinase, casein kinase II, phosphorylates serine residues within the consensus site SXXD/E (Barik and Banerjee 1992). The phosphorylation of P is essential for transcriptional activity. Located between domain I and II is a hypervariable region which may act as a hinge or spacer region to provide correct spatial arrangement of the active domains (Bilsel et al. 1990). Domains II and III are both basic and are located near the carboxy terminus. Domain II functions in transcriptional activation by binding to the polymerase (L). There is a short stretch of amino acid in domain III which is highly basic and is conserved among different strains of VSV. This domain binds to the N-RNA template (Palash et al. 1988). Although the P proteins of various VSV serotypes exhibit low sequence homology, they have a similar distribution of hydropathy. This finding suggests that the overall conformation of P may be conserved and is important to function (Gill and Banerjee 1985).

Unlike other rhabdoviral genes which encode only a single protein, the P gene of VSV and the M1 gene of RV are polycistronic. Herman et al. (1985) described a second protein encoded in the same open reading frame as P, and two small, N-terminally nested proteins encoded in an overlapping ORF in the P gene have been described (Spiropoulou and Nichol 1993). These nested proteins, which have been designated C and C', are highly basic and are localized in the cytoplasm of virus-infected cells. They are not present in purified virions (Spiropoulou and Nichol 1993). In RV, 4 additional proteins were found encoded in the same ORF as P. These proteins were determined to be the result of a leaky scanning mechanism of translation

(Cheniket al. 1995) and were localized in both the cytoplasm and in the nucleus. The role of these protein in viral infection remains uncertain.

The matrix protein

Rhabdoviral matrix proteins are multifunctional (for a review, see Wagner 1987). The matrix protein of VSV is a major structural component of the virion and is thought to be important in virion assembly, viral cytopathic effects (CPE) and inhibition of host cell transcription.

The matrix protein plays an important role in the initiation of virion assembly by forming a bridge between the host plasma membrane (Bergmann and Fusco 1988) and the ribonucleocapsid core (Wilson and Lenard 1981). This interaction promotes the condensation of nucleocapsids into tightly coiled structures (Newcomb et al. 1985),which, after budding is complete, is surrounded by the plasma membrane containing the viral transmembrane glycoprotein.

The matrix protein is the only viral protein found in the cell nucleus in substantial quantity (Lyles et al. 1988) and has been shown to inhibit host cell-directed transcription of target genes in *vivo* (Black and Lyles 1992). Its role in inhibition of host transcription can be separated genetically from its role in virus assembly (Black et al. 1993). Contrary to the effects on transcription, M has been shown to enhance host directed translation in *vivo* (Black et al. 1994).

Studies on temperature sensitive mutants of M and microinjection of M antisense mRNA into infected cells revealed that M alone is responsible for the part of CPE which most likely affects the cytoskeleton and causes cell rounding (Blondel et al. 1990). Melki and coworkers (1994) demonstrated that the highly basic N-terminal domain of M interacts strongly with tubulin both in *vivo* and in *vitro*. This finding suggested that this interaction causes disruption of the microtubular network and subsequent CPE (Melki et al. 1994).

Like P, M is modified by phosphorylation (Imblum and Wagner, 1974) but whether phosphorylation affects function is unknown. The M protein has a highly conserved, basic amino-terminus. Within this domain, a region encompassing amino acids 34 to 50 has been found to interact with the ribonucleocapsid (RNP) core (Ogden 1986). No long hydrophobic stretches suggestive of a transmembrane domain are present (Morita et al. 1987); rather, it is believed that first 10 amino acids of the amino terminus interacts directly with the viral envelope or stabilize a conformation that is required for M protein / membrane interaction (Lenard and Vanderoef 1990, Chong and Rose 1994).

CHAPTER 3
SEQUENCE ANALYSIS OF THE MATRIX PROTEIN GENES OF THE FISH RHABDOVIRUS, INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS

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Abstract

The complete nucleic acid and deduced amino acid sequences of the M1 phosphoprotein and M2 matrix protein genes of infectious hematopoietic necrosis virus (IHNV) were determined from cDNA genomic and mRNA clones. The predicted M1 open reading frame (ORF) encodes a 230 amino acid protein with a estimated molecular weight of 25.6 kDa. Five possible phosphorylation sites were identified with the consensus sequence SXXE/D. In addition, a second ORF located within the M1 was identified which is 146 nucleotides in length with the potential to encode a 42 amino acid protein with a calculated weight of 4.8 kDa. This protein is highly basic and is similar to the small proteins found encoded within the phosphoprotein gene of vesicular stomatitis virus and rabies virus. The predicted M2 ORF is 195 amino acids in length and encodes a protein with a calculated molecular weight of approximately 22 kDa. The amino acid sequence of IHNV M1 and M2 were aligned with other rhabdoviruses to determine the extent of relatedness and to locate conserved regions which may be important to function.

Introduction

Animal rhabdoviruses are presently divided into 3 genera: (1) *Lyssaviruses* or rabies-like viruses, (2) *Vesiculoviruses*, the prototypic member being vesicular stomatitis virus and (3) *Ephemeroviruses*, the prototype member being bovine ephemeral virus. The fish rhabdovirus infectious hematopoietic necrosis virus (IHNV) is classified as a *Lyssavirus* since its genome structure closely resembles that of rabies virus (RV). The single-stranded, negative sense genome of IHNV consists of 6 genes in the order 3' N-M1-M2-G-NV-L 5' (Kurath et al. 1985) with N encoding the nucleocapsid protein; G, the glycoprotein; M1 and M2, the matrix proteins; NV, the nonvirion-associated protein; and L, the RNA-dependent-RNA polymerase. RV M1

and M2 proteins and their counterparts in the *Vesiculovirus* genus, P and M, have been well characterized, whereas little is known about the matrix proteins of IHNV. In order to further characterize this important fish pathogen, the M1 and M2 matrix genes were sequenced and amino acid comparisons were made with other fish *Lyssaviruses* to determine if important functional domains could be identified.

Rhabdovirus matrix proteins have been studied most extensively for VSV and they have been shown to play important roles in viral transcription, replication and virion assembly . In VSV, P (phosphoprotein) is an essential accessory factor for L in viral transcription and replication (see review, Banerjee and Barik 1992). Together they constitute the functional RNA-dependent-RNA polymerase. P is highly phosphorylated and is very acidic, both important for function. P is known to be one of the least conserved proteins in VSV but it does contain 3 distinct functional domains. In VSV and RV, the P gene is the only gene which has been shown to be polycistronic (Herman et al. 1985, Spiropoulou and Nichol, 1993 and Chenik et al 1995).

The RV and VSV matrix proteins, M2 and M respectively, are multifunctional (for a review, see Wagner 1987). During virion assembly M interacts with the ribonucleocapsid core and binds to the cytoplasmic surface of the plasma membrane, leading to a condensation of the nucleocapsids into tightly coiled structures. In VSV, M alone causes the cytopathic effects in cell culture (Blondel et al. 1990) and has been shown to shut down host-directed transcription of target genes in *vivo* (Black and Lyles 1992).

The complete nucleotide sequences of M1 and M2 of IHNV were determined. Putative open reading frames were identified and deduced amino acid sequences were compared with corresponding sequences of other rhabdoviruses. Comparison of the deduced amino acid sequences between two different strains of IHNV (K and Round Butte I) revealed that the M1 and M2 amino acid sequence was well conserved between the two strains. IHNV M1 and M2 also shared considerable sequence

homology with the fish *Lyssaviruses*, viral hemorrhagic septicemia virus (VHSV) and the hirmae rhabdovirus (HRV), but showed little sequence homology with their RV and VSV counterparts. Thus, IHNV, VHSV and HRV are more closely related to each other than to the other rhabdoviruses, VSV and RV. This may indicate a subdivision of the fish rhabdoviruses into a separate subgenera.

Materials and Methods

Description of M1 and M2 clones

Genomic cDNA clones containing the M1 phosphoprotein gene and the M2 matrix protein gene of infectious hematopoietic necrosis virus (IHNV) Round Butte type 1 isolate (RB1) were constructed by Dr. Gael Kurath at the University of Washington. These clones included three full length M1 clones; pIN7, pIN9, pIN11, a partial M1 clone, pIN13 and one full length M2 clone. Additional M2 clones were constructed by reverse transcriptase PCR amplification from total viral and cellular RNA isolated by the RNAzol method (Tel Test, Inc.) using a poly T primer with an adapter and a 5 ' specific M2 primer. Amplified M2 PCR products were cloned directly into the pCRII vector (Invitrogen, San Diego, CA) and transformed into TA One Shot Competent Cells according to the manufacturer's directions (Invitrogen).

DNA sequencing and computer analysis

Double-stranded plasmid templates that were used in sequencing reactions were purified with either Magic Mini-prep columns by Promega or Biorad spin columns. Deoxyoligonucleotide specific sequencing primers were synthesized at the Central Service Facility of the Center for Gene Research and Biotechnology on the Oregon State campus (Table 3.1). Manual sequencing was performed by the Sanger method of

di-deoxysequencing (Sanger et al. 1977) on double-stranded plasmid templates either utilizing the Sequenase (USB, Cleveland, OH), United States Biochemical or Taquence (USB) sequencing kits. Some sequence data were also generated on an ABI 373A (Applied Biosystems) automated DNA sequencer at the Central Service Facility, Oregon State University. Clones were sequenced on both strands using specific or universal primers.

Table 3.1 Deoxynucleotide primers used in sequencing and PCR reactions.

Primer	Sequence 5'-3'	Gene/Location
ME130	CAACAACCCCCTCCTCTCCCG	N (+)*1287-1306
ME131	CGGGTTTGACGGTTTGGCTGTT	G (-) 103-124
ME119	AGCAAGAGCACGAGAAAGTC	M1 (+) 403-422
ME120	TGCCCTTGGATTCGGAAT	M1 (+) 527-544
ME166	GAGACCCGACAAGAACAAGG	M1 (+) 200-219
ME167	AGGATGAGTTGCTCCAGTTG	M1 (-) 228-247
ME192	CAACCATGGCAGATGGAGAAG	M1 (+) 1-51
ME193	GCATGAAGCAAGATCTATAGACATCCT	M1 (-)
ME195	GAGACCATGGCTATTTTCAAGAGAGC	M2 (+) 50-76
ME196	CTGACCTTCGCGGATCCCG	M2 (-)
ME218	GCTCTCTTGAAAATAGACATGCTCTCT	M2 (-)
ME219	GATCTATTACTCCATGGATCTGGCGG	M2 (+) 199-124
ME220	GATCCCCATGGAGACCTACTCAGTC	M2 (+) 412-436
ME221	GTCGGATCCTACATGGCTGGATG	M2 (-)
ME222	GACTACTATGCCCAGGAGACAGCC	M2 (+) 572-595
ME223	CTGTCTTGGACGGAGTGAGGAGTG	M2 (-) 714-737

* denoting positive sense (+) or negative sense (-) primer

Nucleic acid sequences were analyzed and the deduced amino acid sequences were determined by using the Genetic Data Environment software package (GDE version 2.2, beta release by Steve Smith). Chou-Fasman protein secondary structure predictions were generated using the Genetics Computer Group Sequence Analysis Software Package Version 7.2-UNIX (Devereux et al. 1984) of the Genetics Computer Group, Inc. (University of Wisconsin). The nucleotide sequences for the matrix proteins of IHNV (K strain), viral hemorrhagic septicemia virus (VHSV), hirame

rhabdovirus (HRV) spring viremia of carp (SVCV), vesicular stomatitis virus (VSV) and rabies virus (RV) were retrieved utilizing the Nentrez Document Retrieval System Version 1.9. Nucleotide and deduced amino acid sequences of M1 and M2 of IHNV were aligned with corresponding genes of the other rhabdoviruses using the Clustal IV Multiple Sequence Alignment (Higgins et al. 1992) along with manual corrections in the GDE software package. Phylogenetic trees were generated from the amino acid alignments using the DeSoete algorithm (DeSoete 1983), a component of Phylip (version 3.5 release by Joseph Felsentein).

Results

The Intergenic Regions

The intergenic regions are defined between the 3' end of one mRNA and the 5' start of the following mRNA. The N /M1, M1/M2 and M2/G intergenic regions were sequenced from genomic clones, pIN7, pIN9 , pIN11 and pIN13 from the Round Butte, type 1 (RB1) isolate of IHNV. These regions were aligned and compared to other fish rhabdoviruses (Table 3.2).

All of the fish rhabdovirus sequences contained a stretch of 7 (A) residues at the terminus of each gene, which is typical for the *Vesiculoviruses* and *Lyssaviruses*. However, the sequence preceding the stretch of (A) residues is different between the genes of each virus and among the various fish rhabdoviruses. The nucleocapsid protein gene (N) of the K strain and RB1 strain of IHNV both terminate with the sequence AGATAG(A)7. This sequence is also found at the N gene terminus of viral hemorrhagic septicemia virus (VHSV) and the hirame rhabdovirus (HRV) (Tokohiko Nishizawa, personal communication). Unlike the N genes, the termini of the M1, M2 and glycoprotein (G) genes of both strains of IHNV end with the sequence AGACAG$(A)_7$. This is different from the sequence reported for the termini of M1,

M2, and G genes of the VHSV isolates and HRV, which end in the sequence AGATAG$(A)_7$. For IHNV it is likely that both sequences, AGACAG$(A)_7$ and AGATAG$(A)_7$ act as transcriptional termination signals and may direct polyadenylation of viral mRNA.

Table 3.2 Nucleotide sequence alignments of the intergenic regions of various fish rhabdoviruses

Gene Junction	Rhabdovirus Isolate	Intergenic Region		
N/M1	IHNV-RB1	CCTAGATAG	**AAAAAAA**	TGGCACT
	IHNV K	CCCAGATAG	**AAAAAAA**	TGGCACT
	VHS-07-71	AATAGATAG	**AAAAAAA**	CGGCACG
	VHS-MAKAH	TATAGATAG	**AAAAAAA**	CGGCACG
	HIRAME	CTTAGATAG	**AAAAAAA**	TGGCACG
M1/M2	IHNV-RB1	CCAAGACAG	**AAAAAAA**	TGGCACG
	IHNV K	CCAAGACAG	**AAAAAAA**	TGGCACG
	VHS-07-71	CACAGATAG	**AAAAAAA**	CGGCTCG
	VHS-MAKAH	TATAGATAG	**AAAAAAA**	CGGCACG
	HIRAME	CCTAGATAG	**AAAAAAA**	TGGCACG
M2/G	IHNV-RB1	CCAAGACAG	**AAAAAAA**	TGGCACT
	IHNV-K	CCAAGACAG	**AAAAAAA**	TGGCACT
	VHS-07-71	ATTAGATAG	**AAAAAAA**	TGGCACA
	VHS-MAKAH	ATTAGATAG	**AAAAAAA**	TGGTACG
	HIRAME	ATCAGATAG	**AAAAAAA**	TGGCACA
		AGA$^{T}/_{C}$AG	**AAAAAAA**	$^{T}/_{C}$GGCAC

These consensus termination sequences are very different from the *Lyssavirus* TG$(A)_7$ and the *Vesiculovirus* YATG$(A)_7$ and appear to be unique sequences for the fish rhabdoviruses.

The intergenic region immediately after the stretch of 7 (A) residues at the gene junction of N/M1, M1/M2 and M2/G of RB1 were aligned and compared to other fish

rhabdoviruses. The consensus sequence for transcriptional initiation in rabies and VSV is AACA and is generally located before the ATG translational start site. Interestingly, the M2 gene of IHNV lacked this transcriptional initiation consensus sequence. In fact the only sequence that all the fish rhabdoviruses share is GCAC, located at position + 3 relative to the poly A residues after each gene. The exception to this location is in the Makah strain (Benmansour et al. 1994) of VHSV, where the GCAC is located at position 17 relative the 5' end of the M1 gene. The GCAC transcriptional initiation site for IHNV is supported by primer extension studies on the NV gene of IHNV where the transcriptional start site was determined to be GCAC (Peter Chiou, 1995 submitted).

The M1 phosphoprotein

The entire nucleotide sequence of the M1 gene was determined by di-deoxysequencing analysis of the IHNV genomic clones, pIN7, pIN9, pIN11 and pIN13. The M1 gene excluding the poly (A) sequence is 762 nucleotides in length (Figure 3.1). The first ATG is located 36 nucleotides away from the end of the N gene, and is presumed to be the start of the M1 open reading frame. This open reading frame encodes a 230 amino acid protein with a calculated molecular weight of 25.6 kDa. The termination codon TGA, at position 726 is located 34 nucleotides upstream of the poly (A) sequence.

Unlike RV and VSV whose M1 proteins have isoelectric points (pI) of 4.36 and 4.84 respectively, the M1 protein of IHNV is a basic protein with an estimated pI of 8.4. This is similar to what has been reported for the M1 protein of VHSV, which has a pI of 9.89 for strain 07-71 and 9.46 for strain Makah (Benmansour et al. 1994). The M1 amino acid sequence of the RB1 strain contains 5 potential phosphorylation sites with the consensus sequence SXXD/E. These sites are concentrated mostly within the first half of the amino acid sequence. The same sites are conserved in the K strain

Figure 3.1 Nucleotide and deduced amino acid sequence the IHNV (RB1) M1 phosphoprotein. The nucleotide sequence is presented in the positive-strand sense as DNA with the deduced amino acid sequence in letter code illustrated below. Nucleotide position is indicated in left margin and amino acid residue is indicated in the right margin. Potential phosphorylation sites with the consensus sequence SXXD/E are underlined. Asterisk * denotes the stop codon.

```
1     atgtcagatggagaaggagaacagttcttcgatctagaaggcgaagacatactgaggcta
      M  S  D  G  E  G  E  Q  F  F  D  L  E  G  E  D  I  L  R  L    20

61    gaatcccgcctgaaaaccccacggaatgacgggcaaatcggcaagaaccccaggcaacgg
      E  S  R  L  K  T  P  R  N  D  G  Q  I  G  K  N  P  R  Q  R    40

121   aaggaggaccaggtgcctcgagagggaccaaagaaagccaccaagagacccgacaagaac
      K  E  D  Q  V  P  R  E  G  P  K  K  A  T  K  R  P  D  K  N    60

181   aagggtctatctcaactggagcaactcatcctaaagtacgttgaggaggagagctgtcag
      K  G  L  S  Q  L  E  Q  L  I  L  K  Y  V  E  E  E  S  C  Q    80

241   gatgccctgaaggacttcggaggtctaatagccaacatcagacaggcccaccaggccgaa
      D  A  L  K  D  F  G  G  L  I  A  N  I  R  Q  A  H  Q  A  E   100

301   ctgacatctcacctagaaaaggttgctacggagcaccgagccaatcttcaggctcttaca
      L  T  S  H  L  E  K  V  A  T  E  H  R  A  N  L  Q  A  L  T   120

361   aagtcccagcaagagcacgagaaagtctcgaaggagattttgtctgcggtaattgctatt
      K  S  Q  Q  E  H  E  K  V  S  K  E  I  L  S  A  V  I  A  I   140

421   cggtccaacctcaacgagaaccacagtcccctgcccaagccactcgacccggatcaggtg
      R  S  N  L  N  E  N  H  S  P  L  P  K  P  L  D  P  D  Q  V   160

481   aaggccgctcgtgcccttggattcggaattgggtatcgaacggccctcaatgtcttcgac
      K  A  A  R  A  L  G  F  G  I  G  Y  R  T  A  L  N  V  F  D   180

541   cgaatcaagggagtcaccccagacaacgcaggatcccaagaggtgaagaacatggccact
      R  I  K  G  V  T  P  D  N  A  G  S  Q  E  V  K  N  M  A  T   200

601   cgggcagcggaggaggacgaatacgagggaagtcccactttcttcaggagggtgatagac
      R  A  A  E  E  D  E  Y  E  G  S  P  T  F  F  R  R  V  I  D   220

661   tccgtaaagaagcgcatgaagcaaggtcaatag
      S  V  K  K  R  M  K  Q  G  Q  *
```

Figure 3.1

Figure 3.2 Alignment of deduced amino acid sequences of IHNV (RB1) , HRV and VHSV (07-71) phosphoproteins. Sequences were aligned using Clustal IV alignment option of the GDE computer package. Potential phosphorylation sites are underlined. Asterisk *denotes identical amino acid residues.

```
        * * *                       *       *
IHNV    MSDGEGEQFF DLEGEDILRL ESRLKTPRND GQIGKNPRQR KEDQVPREGP
HRV     MSDNEGEQFF DIPKNALDRV EARTMCPRED GKVVRKQAPL KEEPRLEAEQ
VHSV    MADIEMSESL VLSHGSLADL DKRLDNAPKD NRSALFSST- -------SGS
        |           |           |           |           |
        1          11          21          31          41

                  *       **        *        **   ** *    *   **
        KKATKRPDKN KGLSQLEQLI LKYVEEESCQ DALKDFGGLI ANIRQAHQAE
        KRSPKKQEKP RGMLPLEQLV LKYVVVVCSL DALREFGGLI AQIRQSHQAD
        TRQKSSSKKK PNPTTLEEII GHFVPEDLQL DATKAFGQLL RRIKMSHQEE
        |           |           |           |           |
       51          61          71          81          91

         * *** *      **   **   ** *  *     ***  *   *    ** *
        LTSHLEKVAT EHRANLQALT KSQQEHEKVS KEILSAVIAI RSNLNENHSP
        MTRHLEAVAT EHRANLQALT KSQQEHEKVS KEILSAVISI RSNLNENSSP
        LTQHLEKVNR GKRAKMGALL ESQKENGKKT DNILSILIAM RGEGAENASK
        |           |           |           |           |
      101         111         121         131         141

           * ** ***    *****   *  **             *        **  *
        LPKPLDPDQV KAARALGFGI GYRTALNVFD RIKGVTPDNA GSQEVKNMAT
        RHKPLDLDQV NAERALGFGV GYRTALNVFG KLRGITPEEA GSQEVKNMAI
        KPKVLDGDQV RNERALGFNR GLTTAAIAMK KFKLEDPLAL CKGSVKRAAL
        |           |           |           |           |
      151         161         171         181         191

         * *  ** *         *     *
        RAAEEDEYEG SPTFFRRVID SVKKRMKQGQ
        REAEEDEYEG SRSFFKKVLD MVKKTMR
        SAMEKEEYDG ERETYSTVSK AIKADVDKLE
        |           |           |
      201         211         221
```

Figure 3.2

isolate of IHNV. No sites were found with the consensus TXXD/E which had been reported for the M1 phosphoprotein of VHSV (Benmansour et al. 1994).

The following phosphoprotein amino acid sequences were aligned: IHNV RB1 and K strains, VHSV 07-71 and Makah strains, HRV, RV, VSV and the plant rhabdovirus, sonchus yellow net virus (SYNV) and percent amino acid similarity and identity were determined. Between the two strains of IHNV, there are only 10 amino acid differences. There was little amino acid identity between IHNV and rabies or IHNV and VSV. Comparisons between the two fish rhabdoviruses, IHNV and HRV indicate that they share a 77% similarity and a 63% identity at the amino acid level (Table 3.3)

Table 3.3 Percentage similarity and identity of amino acid sequences between IHNV (RB1) and other rhabdovirus phosphoproteins.

IHNV (RB1)	IHNV(K)	HRV	VHSV	RV	VSV	SYNV
Percentage similarity	98	77	52	40	43	40
Percentage identity	94	63	38	17	19	16

A second overlapping open reading frame within the M1 gene is located at position 121 from the end of the polyadenylated sequence of the N gene. This second open reading frame is 146 nucleotides in length and has the potential to encode a 42 amino acid protein with an estimated molecular weight of 4.8 kDa. This putative protein is highly basic with an estimated isoelectric point of 12.8 and is rich in arginine (9 residues). The putative protein exhibits no sequence homology with other known rhabdovirus proteins, but does share the common characteristics of arginine richness and basic pI with that of the proteins encoded within the phosphoprotein genes of RV (Chenik et al. 1995) and VSV (Spiropoulou and Nichol, 1993). These basic proteins

have been found in infected cells but are not associated with the virion. This potential to encode a small protein with similar characteristics is also found in the M1 gene of VHSV.

The M2 matrix protein

The M2 gene sequence was determined for three genomic clones, pIN7, pIN9 and pIN11. Only pIN7 contained the complete M2 gene and additional clones were generated from mRNA to confirm the nucleotide sequence. The clones pCRII-M2593 and pCRII-M2-770 were constructed by reverse transcriptase PCR of viral mRNA.

The M2 gene is 739 nucleotides in length with the first ATG translational start site located at position 56 from the 5' end of the M1 gene (Figure 3.3). The putative open reading frame is 585 nucleotides in length and encodes a protein of 195 amino acids with an estimated molecular weight of 21.8 kDa. The TAG termination codon is located at position 641, 98 nucleotides upstream of the M2 gene poly (A) sequence. This is the longest 3' noncoding region of all the IHNV genes.

The IHNV M2 protein is highly basic, with an estimated isoelectric point of 10.08. This is a common characteristic of rhabdoviruses. For the fish rhabdovirus VHSV, the calculated pIs were 10.07 for the 07-71 strain and 10.23 for the Makah strain (Benmansour et al. 1994).

The alignment of the matrix protein amino acid sequences for IHNV (RB1), IHNV (K), HRV, VSV (Indiana strain), RV (ERA strain), VHSV (Makah and 07-71 strains) and SVCV was carried out to determine the extent of relatedness. The IHNV RB1 and K strains differed by only 4 amino acids. The deduced IHNV M2 amino acid sequence shared no significant homology with either VSV, RV or the fish *Vesiculovirus*, SVCV. IHNV and HRV M2 proteins shared a 74% amino acid identity and IHNV and VHSV M2 proteins exhibited a 37% amino acid identity (Table 3.4) with three localized regions of homology (Figure 3.4). The highest sequence

Figure 3.3 Nucleotide and deduced amino acid sequence of the IHNV (RB1) matrix protein. The nucleotide sequence is presented in the positive-stand sense as DNA. The deduced amino acid sequence in letter code is illustrated. Nucleotide position is indicated in the left margin and amino acid residue is indicated in the right margin. Asterisk * denotes the stop codon

```
1     atgtctattttcaagagagcaaagagaacagttctgatccctccttctcacctcctcagc
      M  S  I  F  K  R  A  K  R  T  V  L  I  P  P  S  H  L  L  S   20

61    ggagacgaggagagggtgacaatactcagtgcagaggggagatcaaggtgactggaaga
      G  D  E  E  R  V  T  I  L  S  A  E  G  E  I  K  V  T  G  R   40

121   accacaagaccacttgaggagaagatctattactctatgaatctggcggctgccatcgta
      R  P  T  T  L  E  E  K  I  Y  Y  S  M  N  L  A  A  A  I  V   60

181   gggggagatctacacccctcattcaaatccatgacctatctgttccagaaagagatggag
      G  G  D  L  H  P  S  F  K  S  M  T  Y  L  F  Q  K  E  M  E   80

241   ttcgggagcacccaagaaaaagtcaacttcggctctcggaaaccagcacctcagaccacc
      F  G  S  T  Q  E  K  V  N  F  G  S  R  K  P  A  P  Q  T  T  100

301   taccaggtgatgaaggcgagggaagtctaccttcagacccagcctctcgagaagaagatc
      Y  Q  V  M  K  A  R  E  V  Y  L  Q  T  Q  P  L  E  K  K  I  120

361   ccaatgcagacctactcagtcagcacagaggggctaccatcaccttcacaggacggttc
      P  M  Q  T  Y  S  V  S  T  E  G  A  T  I  T  F  T  G  R  F  140

421   ctcttttcatccagccatgtaggttgtgacgacaacaggaccaaactggcggggcttgat
      L  F  S  S  S  H  V  G  C  D  D  N  R  T  K  L  A  G  L  D  160

481   gggttcacaacctccgacagctaccagagggtcaaagactactatgcccaggagacagcc
      G  F  T  T  S  D  S  Y  Q  R  V  K  D  Y  Y  A  Q  E  T  A  180

521   ctggccctgaccttcgccgctcccgaaaagcgggggaaggaaaaatag
      L  A  L  T  F  A  A  P  E  K  R  G  K  E  K  *
```

Figure 3.3

Figure 3.4 Alignment of deduced amino acid sequences of IHNV (RB1) HRV and VHSV (07-71) matrix proteins. Sequences were aligned using Clustal IV alignment option of the GDE computer package. Asterisk * denotes identical amino acid residues.

```
      *  *** * *  * ** ** *  ** **       **     *   *     **
IHNV  MSIFKRAKRT VLIPPSHLLS GDEERVTILS AEGEIKVTGR RPTTLEEKIY
HRV   MSLFKRTKKT ILIPPPHLLS GDEDRVTVLN AEGEIKISGD RPTTLDEKIY
VHSV  MALFKR-KRT ILVPPPHLTS NDEDRVSTIL TEGTLTITGP PPGNQVDKIC
      |          |          |          |          |
      1         11         21         31         41

        * ** **    * ** *      ***  *  ** * **  * * *     *
      YSMNLAAAIV GGDLHPSFKS MTYLFQKEME FGSTQEKVNF GSRKPAPQTT
      YSMSLAAAIL GGNLHPSFQS LTYLFQQEME FGSTIEKVNF GSRKPAPLTP
      MAMKLARAIL CEDQHPAFNP LVHLFQSAMI FGETSEKIDF GTRSKTLITS
      |          |          |          |          |
     51         61         71         81         91

                       *    *     *          *  * *  *   ***
      YQVMKAREVY LQTQPLEKKI PMQTYSVSTE GATITFTGRF LFSSSHVGCD
      IRWQKPEKCS FRPQPLDKKI PPQIYTVSVE GATITFSGRF LFSASHVGCD
      FKIAEAKAIY LDSSPVRSRI EAKKYTTPIR HGSVTYYGPF IFADDHVGGK
      |          |          |          |          |
    101        111        121        131        141

        * **  *  **  *  *    ****  * *          *     *
      DNRTKLAGLD GFTTSDSYQR VKDYY--AQE TALALTFAAP EKR-G----K
      DNRVKLAGLD GFITSPNYQR VKDYY--AQE TVLALSFEIP TKK-G----K
      GHREKLGALC GFLQSGPYGQ AKDYYNRAVE EEIGIPPRDP KRRSGTSSVR
      |          |          |          |          |
    151        161        171        181        191

      EK
      PW
      |
    201
```

Figure 3.4

conservation occurred within the first 29 amino acids. This region contained a stretch of basic residues, a feature which has been described for the amino termini of RV and VSV and is thought to play a role in inhibition of host transcription.

Table 3.4 Percentage similarity and identity between the amino acid sequences of IHNV M2 and other rhabdovirus matrix proteins.

IHNV(RB1)	IHNV(K)	HRV	VHSV	RV	VSV	SVCV	SYNV
Percentage similarity	99	87	60	42	41	37	37
Percentage identity	98	74	39	17	20	16	21

Discussion

RNA viruses are known to have a high rate of evolution (Tordo et al. 1992) and within the *Lyssavirus* and *Vesiculovirus* genera certain genes are more conserved than others. The relative amino acid conservation of the virion proteins of mammalian lyssaviruses is nucleoprotein > matrix protein > glycoprotein > phosphoprotein (Bourhy et al. 1993). The polymerase gene was not included in this study but is considered to be one of the most highly conserved gene among the *Rhabdoviridae* (Tordo 1992). The results of the amino acid comparisons between the matrix protein of IHNV and VHSV support these observations. Between the two strains of IHNV, M2 is more conserved than M1 and among IHNV, HRV and VSHV, M2 is more highly conserved (Table 3.3 and 3.4). These findings are consistent with immunoblot studies in which IHNV sera reacted with VHSV viral proteins N, G and M2 but not M1 (Nishizawa et al. 1991).

The function of the phosphoprotein as a transcriptional activator is thought to be maintained by structural similarities rather than by sequence similarities (Gill and

Banerjee 1985, Bourhy et al. 1993). The phosphoproteins of RV and VSV share a highly hydrophilic and variable region which contains most of the putative phosphorylation sites (Tordo et al. 1986). Although IHNV and VHSV phosphoproteins are basic proteins, unlike the acidic phosphoproteins of RV and VSV, they also have a hydrophilic, variable region which contains the potential phosphorylation sites (Figure 3.5 and Figure 3.2). The phosphoproteins of IHNV, VHSV and HRV have a conserved region immediately following this variable region, which may correspond to the conserved N-RNA binding domain found in the phosphoprotein of VSV (Gill et al 1986) and RV (Tordo et al. 1986).

The IHNV matrix protein is similar to all reported rhabdovirus matrix proteins; it is highly basic and contains a high content of charged amino acids concentrated at the amino terminus. A highly charge amino terminus has been found in the *Vesiculoviruses* and the *Lyssaviruses* (Rose and Gallione 1981 Bourhy et al 1993) and in paramyxoviruses (Chambers et al. 1986). In VSV the first 51 amino acids are required for stable interaction with the plasma membrane (Chong and Rose, 1994), viral assembly (Black et al. 1993) and possibly in the inhibition of RNA transcription (Ogden et al. 1986). This region is also conserved among IHNV, HRV and VHSV. The matrix protein of VSV interacts with the host cell plasma membrane (Bergman and Fusco 1988); however, a long hydrophobic stretch of amino acids is not present (Morital et al. 1987). The IHNV M2 protein is also lacking a long stretch of hydrophobic residues (Figure 3.6).

The phosphoprotein genes of many single-stranded, negative sense RNA viruses have been shown to encode more than one protein (Lamb et al. 1981, Giorgi et al. 1983 and Bellini et al. 1985). The phosphoprotein of IHNV has the potential to encode a small, highly basic protein in a second reading frame; a protein with similar characteristics is encoded within the VHSV phosphoprotein gene. For both RV and VSV, additional proteins encoded within the phosphoprotein gene are not associated

Figure 3.5 Predicted secondary structure of IHNV M1 (RB1). Chou-Fasman secondary prediction was generated using the GCG computer software package. Hexagons denoted hydrophilic regions, while diamonds indicate hydrophobic regions. Rapid zigzags are beta sheets, undulating straight lines represent random coils, and sine waves are alpha helices.

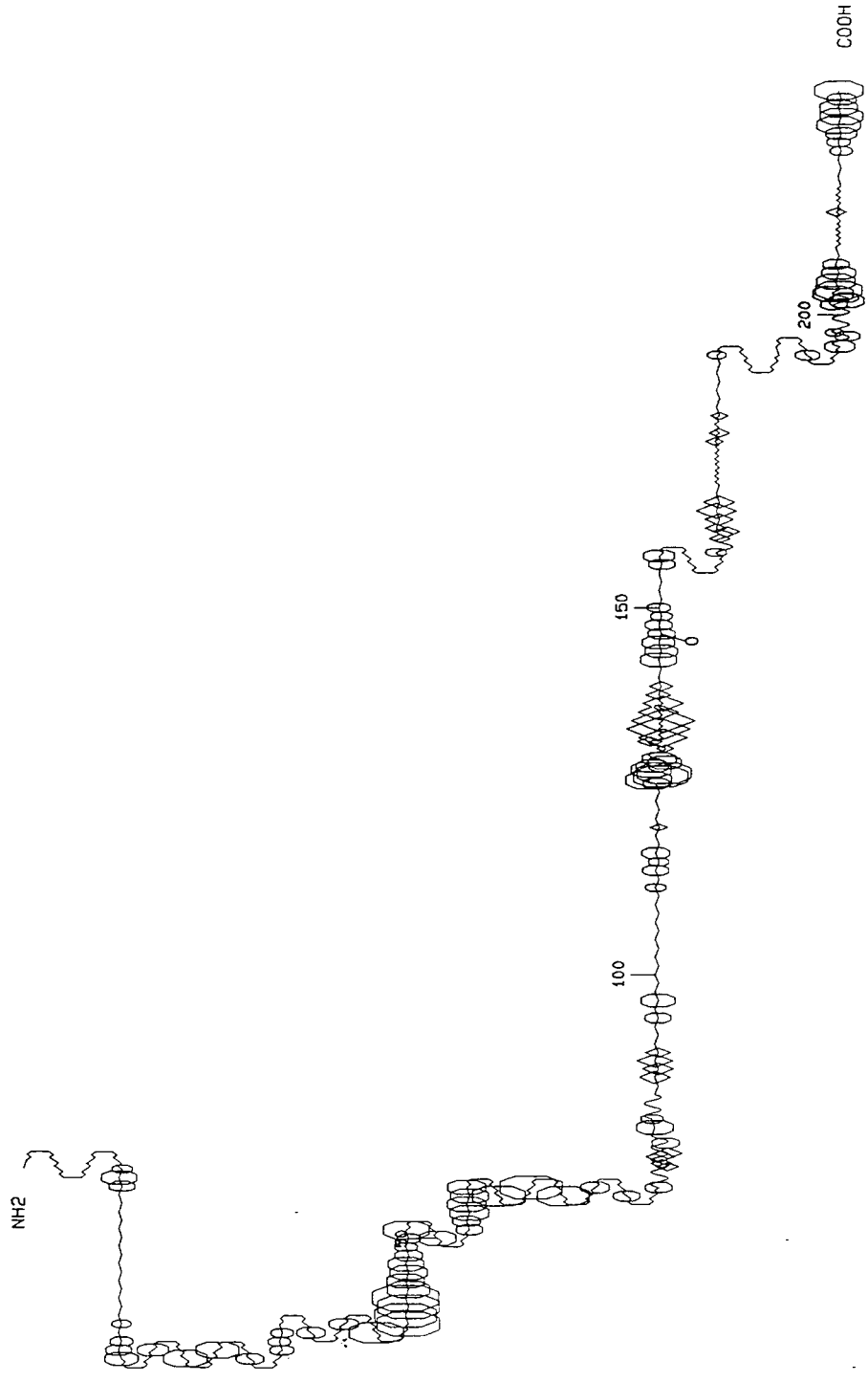
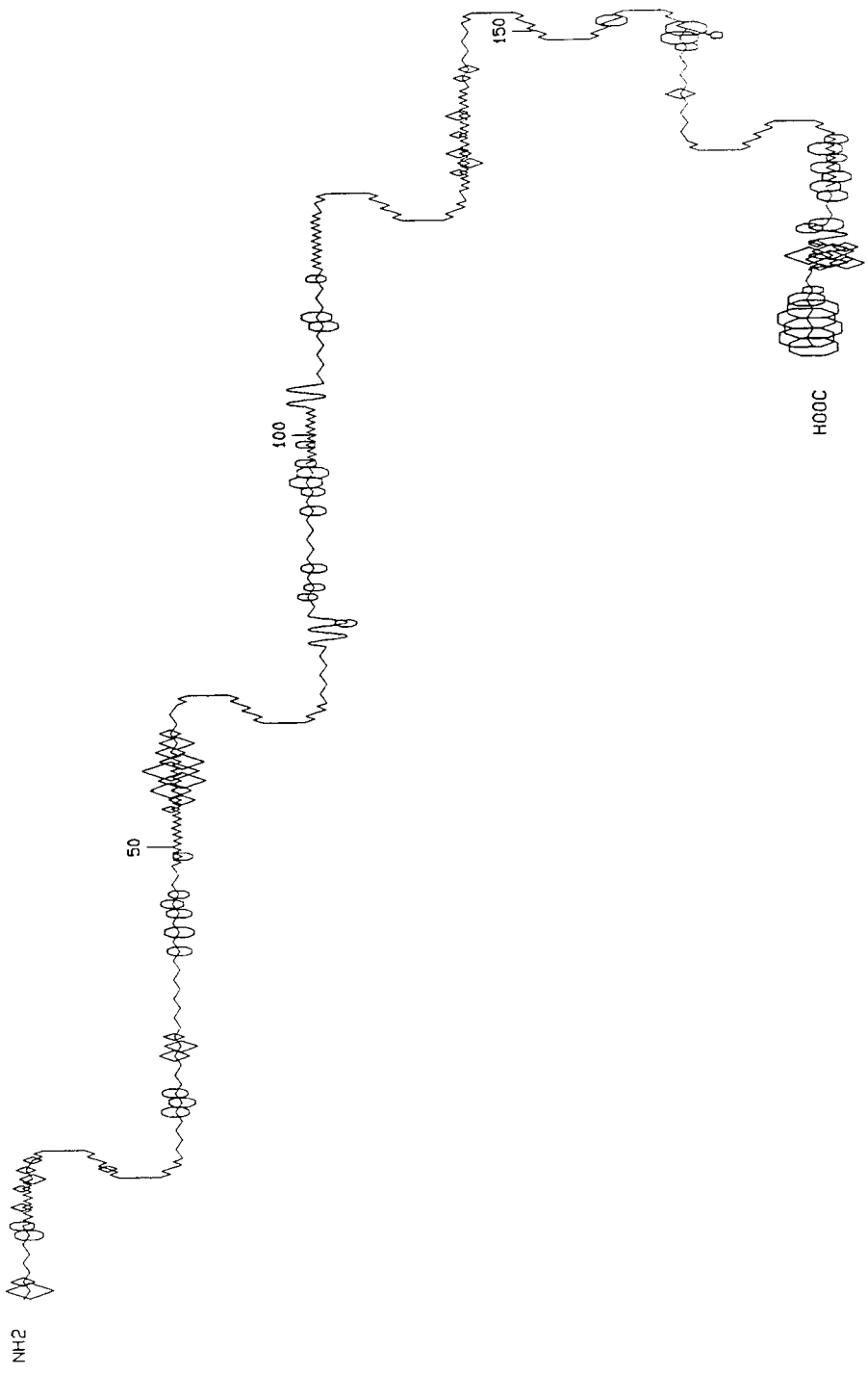


Figure 3.5

Figure 3.6 Predicted secondary structure of the IHNV M2 (RB1). Chou-Fasman secondary prediction was generated using the GCG computer software package. Hexagons denoted hydrophilic regions, while diamonds indicate hydrophobic regions. Rapid zigzags are beta sheets, undulating straight lines represent random coils, and sine waves are alpha helices.



Figrure 3.6

with the mature virion, but have been localized in the cytoplasm and nucleus of infected cells. This finding suggests that these proteins may participate in RNA synthesis. Recent studies on the phosphoprotein of the Sendai paramyxovirus suggest that these proteins are involved in the inhibition viral mRNA synthesis . It is not known whether the second ORF in the M1 gene of IHNV is utilized. To answer this question, this gene has been cloned into a protein fusion vector to provide sufficient quantities of the putative protein for antibody production. Until that immunological reagent is available, studies on the expression of the M1-second ORF cannot be made.

The nucleotide and deduced amino acid sequences for the M1 and M2 matrix proteins of IHNV were determined. Comparison of the intergenic regions of the IHNV, VHSV and HRV indicated that the consensus sequence AGA$^{T}/_{C}$AG $(A)_7$ $^{T}/_{C}$GGCAC is unique to these fish lyssaviruses. Calculations of the amino acid similarity and identity based upon the amino acid alignments between IHNV and other rhabdoviruses were carried out. These findings indicated that IHNV is more closely related to HRV and VHSV than to either VSV or RV. Phylogentic trees generated from the amino acid sequence alignments support this finding (Figure 3.7 and 3.8) in which the branching divides the fish *Lyssaviruses* from the *Vesiculoviruses* and *Lyssaviruses*.

Figure 3.7 The phylogenetic tree for the phosphoproteins of the rhabdoviruses. Deduced amino acid sequences of the phosphoprotein genes of the IHNV RB1and K strains, VHSV Makah and 07-71 strains, HRV, VSV Indiana strain, RV ERA strain and the plant rhabdovirus, sonchus yellow net virus (SYNV) were aligned using the Clustal IV Multiple Sequence Alignment. Phylogentic trees were generated from the amino acid alignments using the DeSoete algorithm, a component of Phylip. SYNV was used as an outgroup.

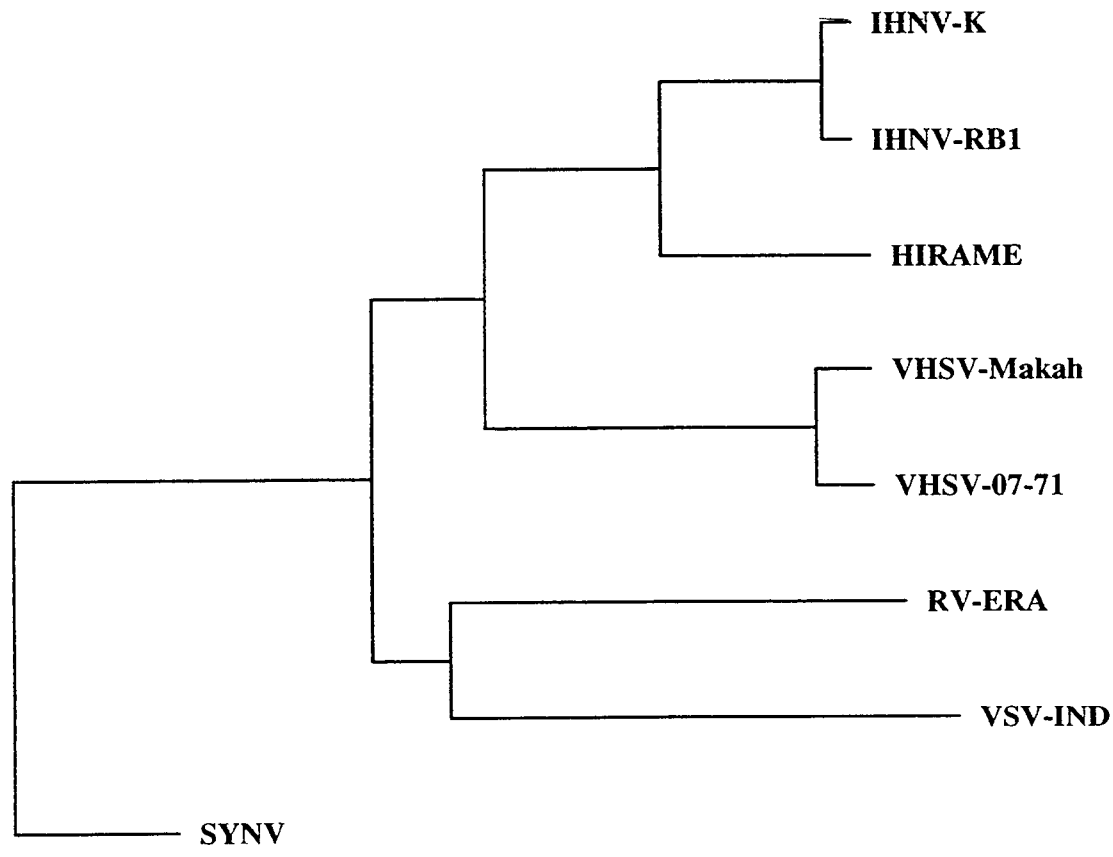

IHNV-K
IHNV-RB1
HIRAME
VHSV-Makah
VHSV-07-71
RV-ERA
VSV-IND
SYNV

Figure 3.7

Figure 3.8 The phylogenetic tree for the matrix proteins of the rhabdoviruses. Deduced amino acid sequences of the matrix protein genes of the IHNV RB1 and K strains, VHSV Makah and 07-71 strains, HRV, VSV Indiana strain, RV ERA strain, spring viremia of carp virus (SVCV) and the plant rhabdovirus, sonchus yellow net virus (SYNV) were aligned using the Clustal IV Multiple Sequence Alignment. Phylogentic trees were generated from the amino acid alignments using the DeSoete algorithm, a component of Phylip. SYNV was used as an outgroup.

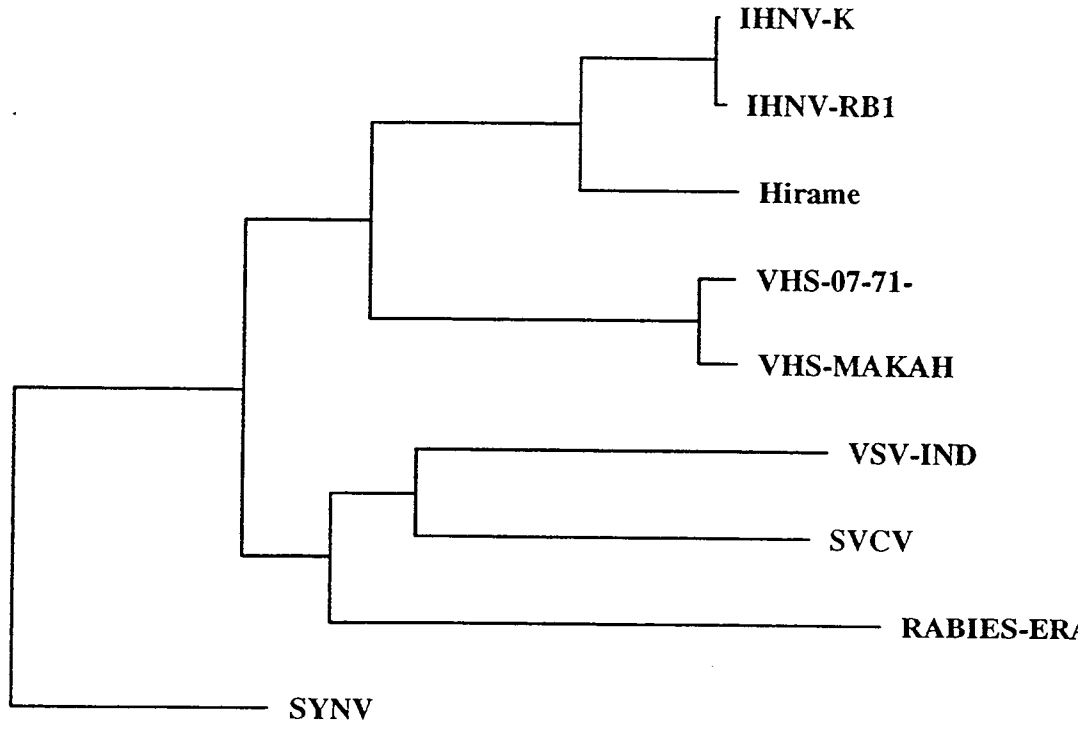

IHNV-K
IHNV-RB1
Hirame
VHS-07-71-
VHS-MAKAH
VSV-IND
SVCV
RABIES-ERA
SYNV

Figure 3.8

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CHAPTER 3
HUMORAL IMMUNE RESPONSE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) FOLLOWING INJECTION WITH PLASMIDS ENCODING THE M1 AND M2 PROTEIN GENES OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS

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Abstract

Plasmids encoding the M1 or M2 matrix protein genes of the fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV) under the control of a viral promoter/enhancer were tested for their ability to elicit an antibody response in rainbow trout (*Oncorhynchus mykiss*) after injection of the DNA into skeletal muscle. The ability of each plasmid to produce antigenically accurate M1 and M2 proteins was initially verified by transcription/ translation reactions *in vitro*. The injected fish were bled at 2, 4 and 6 weeks postinjection, and the production of antibody to M1 and M2 in rainbow trout was monitored by immunoblotting. IHNV-specific antibody titers in fish sera were monitored over a 6 week period by enzyme-linked immunosorbant assay (ELISA). No antibody was detected within that period. However, this was not unexpected since these proteins are not transferred to the cell surface. This work may prove useful in the development of an effective DNA-based subunit vaccine against IHNV.

Introduction

Previous studies have reported successful genetic immunization against viral pathogens (Ulmer et al. 1993, Xiang et al. 1992). Upon injection, naked DNA is taken up by cells and the DNA-encoded protein is synthesized within cells. Since the protein of interest is endogenously expressed, it is thought to be able to elicit both humoral and cell-mediated immune responses. Immune responses to DNA vaccines have been measured by the presence of specific antibodies and T-cell cytotoxic responses.

Preliminary studies in rainbow trout by Anderson et al. (1995a) described important parameters such as the route of injection and the identification of the cytomegalovirus immediate early enhancer/promoter (CMV IEP) as the promoter which allowed the highest level of expression for extended periods of time. In these

studies, the injected DNA was shown to persist as an unintegrated, non-replicated plasmid DNA and protein expression occurred within the cells of the skeletal muscle and other organs. More recently, Anderson and coworkers (Anderson et al. 1995b) demonstrated the effectiveness of a DNA-based vaccine against IHNV in which the glycoprotein (G) gene was used to genetically immunize rainbow trout. Protection against subsequent challenge with IHNV was augmented when two plasmids encoding G and nucleocapsid protein (N) were used in single vaccine. These results suggest that combinations of viral proteins may afford enhanced protection.

Neutralizing antibodies are only one line of protection against a viral disease and the other arm of the immune system, the cellular immune response, is also important.. Genetic immunization in mice with a plasmid carrying the rabies virus glycoprotein resulted in the development of rabies glycoprotein- specific cytolytic T cells, lymphokine-secreting T helper cells, and rabies virus -neutralizing antibodies (Xiang et al. 1994). Animals were effectively protected against subsequent rabies virus (RV) challenge. In addition, protection against rabies in mice has been achieved by immunizing with the ribonucleoprotein complex (Dietzschold et al. 1987). This complex includes the nucleoprotein as an internal protein that does not induce neutralizing antibody response but may elicit a cytotoxic T cell response. Cytotoxic T cell responses are directed against the glycoprotein of RV and are thought to play a role in protection against disease (Kawano et al. 1990) and are also made against the phosphoprotein of RV (Larson et al. 1992).

Preliminary experiments were carried out with plasmids encoding the IHNV phoshoprotein (M1) and matrix protein (M2) determine if specific antibody responses could be elicited in fish after DNA injection. Plasmid constructs encoding M1 and M2 were injected directly into rainbow trout and serum antibody levels to the M1 and M2 proteins were monitored by enzyme-linked immunosorbant assays and

immunoblotting. No detectable levels of antibodies could be measured either by immunoblotting or ELISA-based assays.

Methods and Materials

Plasmid constructs

The M1 and M2 genes of IHNV RB1 were cloned into the eukaryotic expression vector, pCR3 (Invitrogen) under the control of the cytomegalovirus intermediate early promoter (CMV-IEP). The complete M1 gene excluding the last 5 amino acids was amplified by the polymerase chain reaction (PCR) using the cDNA clone pIN7. Amplified M1 PCR products were ligated directly into the pCR3 vector and ligations reactions were transformed into Invitrogen ONE Shot TOP10F' competent cells utilizing the Eukaryotic TA Cloning Kit (Invitrogen). M1 clones were screened for the correct orientation by digesting with BamHI (Promega). A plasmid containing the M2 gene, pCRII-M2770 was used to subclone M2 into pCR3. The plasmid pCRII-M2770 was restricted with EcoRI to excise the M2 insert. The insert was isolated in a 1.5% low melting temperature gel, excised and recovered using the Gene Clean Kit (Bio 101, Inc). The M2 fragment was ligated into pCR3 vector which had been restricted with EcoRI and treated with calf intestinal alkaline phosphatase (CIAP) (Promega). Ligation reactions and transformation into competent ONE Shot TOP10F' cells (Invitrogen) were carried out according to the manufacturer's instructions. Plasmid DNA was purified using the Qiagen affinity chromatographic columns (Qiagen, Inc.) and the quantity determined in a Varian DM-80 spectrophotometer at 260 nm.

In vitro-transcription

The plasmids pCR3-M1 and pCR3-M2770 were used as templates in *in vitro* transcription reactions to synthesize uncapped, runoff T7 mRNAs. Plasmids were linearized by restriction digest with PstI (Promega) and DNA was isolated by agarose gel electrophoresis. Linear products were excised and recovered from the gel using the Gene Clean II Kit (Bio 101, Inc.) and the quantity of DNA was determined spectrophotometrically. *In vitro* transcription reactions were carried out using a protocol for the synthesis of large quantities of RNA by Promega. Briefly, the 3' overhang resulting from the Pst I digestion was converted to a blunt end by adding all components of the transcription reaction except the nucleotides and RNA polymerase. Klenow DNA polymerase was added at a concentration of 5u/ug of DNA, and reactions were incubated for 15 minutes at 22°C. Transcription was initiated by the addition the ribonucleotides and the T7 RNA polymerase (Promega) and reactions were incubated for 120 minutes at 37°C. Following transcription, RNA was extracted 2 times with an equal volume phenol chloroform and precipitated by the addition of 1/10 volume of 3M NaOAc and 3 volumes of 95% ethanol. *In vitro* transcribed RNA was recovered by high speed centrifugation for 20 minutes. RNA pellets were rinsed 2X with 70% ethanol, air dried and resuspendend in Tris -EDTA (TE) buffer, pH 8.0.

In vitro translation

Translation of *in vitro* transcribed mRNA was carried out in a rabbit reticulocyte lysate system (Promega). Briefly, isolated RNA was translated *in vitro* following the Standard Reaction protocol with [^{35}S] methionine as the radioactive label. Protein products were spun through a Centricon #3 filter at 7000g for 1 hour to concentrate the samples and remove unincorporated label.

Gel electrophoresis and immunoblotting of proteins

Concentrated, *in vitro* -synthesized proteins were analyzed by polyacrylamide gel electrophoresis (PAGE) as follows. Protein samples were diluted in an equal volume of 2X loading buffer and run on a 12.5% gel with a 5% gel stacker at 50 V, until the tracking dye reached the bottom of the gel. IHNV-infected chinook salmon embryo cell (CHSE-214) extract labeled with [^{35}] S methionine was run in parallel as a molecular weight marker . Labeled, uninfected CHSE-214 cell extracts were run as a negative control. Gels were dried onto Whatman paper under vacuum and exposed on Kodak film for 48 hours. For immunoblotting, *in vitro* translation products were subjected to PAGE and transferred onto nitrocellulose membranes (Shleicher and Schuell) at 50 V for 45 min. Blots were probed with 1/200 rabbit anti-IHNV Ig in Tris buffered saline (TBS, 20mM Tris base, 500 mM NaCl, pH 7.5) for 1 hour. Membranes were rinsed with TBS and 1/1000 phosphatase-labeled, goat anti-rabbit Ig (BioRad) was added and blots were incubated for 1 hour. Membranes were rinsed in TBS pH 9.2. Blots were developed with a one component phosphatase substrate (Kirkegaard and Perry) in the dark.

Transfection of EPC fish cells

Epithelioma papulosum cyprini (EPC) cells from the common carp *Cyprinus carpio* (Fijan et al. 1983) were propagated in minimum essential medium (MEM) containing 10% fetal bovine serum, 2% penicillin-streptomycin (1 unit/ug), 2% fugizone, 2% 200 mM L-glutamine, 1% gentamycin (50 mg/ml), buffered with 1M Tris buffer to pH 7.0. EPC cells were grown to confluency in 6 well tissue culture plates (Corning, 9.4 cm^2/well) and used in DNA transfection studies. For each transfection 1 ug of either pCR3-M1 or pCR3-M2770 was diluted in 100 ul of OPTI-MEM I Reduced Serum Medium (GIBCO-BRL) This was then added to another solution containing 6

ul Lipofectamine reagent (GIBCO-BRL) in 100 ul OPTI-MEM I Reduced Serum Medium and incubated at room temperature for 30 minutes. During incubation, EPC cells were rinsed 1 time with serum free medium. For each transfection, 0.8 ml of serum free medium was added to the solution containing the DNA and Lipofectamine. The DNA-Lipofectamine solution was then added to the cells and the treated cells were placed at 20°C in an incubator culture chamber (C.B.S. Scientific C.: model no. M-625) containing a blood gas mixture of 9.9% mol/mol carbon dioxide, 10.2% mol/mol oxygen and 70.9% mol/mol nitrogen (Airco medical gases BOC). After a 24 hour incubation, medium was removed from each well and replaced with 2X MEM with 10% fetal bovine serum and incubated for five days as described above.

Immunocytochemical staining of transfected cells

Alkaline immunocytochemical staining was carried out on transfected EPC cells following the procedure described by Drolet 1993. Cells were fixed and stained with crystal violet in formalin (25% formalin, 10% ethanol, 5% acetic acid, 1% w/v crystal violet) for 1 hour, rinsed with water and destained with 70% ethanol. Fixed cells were equilibrated by adding phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCL, 4.3 mM $NaHPO_3.7\ H_2O$, 1.4 mM $KHPO_4$), pH 7.4 and incubating at room temperature with gentle rocking. Cells were blocked in a solution of 5% nonfat powdered milk (Saco Foods, Madison, WI) in PBS for 1 hour, media was removed and rabbit anti-IHNV antibody was added at dilutions of 1/50, 1/100 or 1/200 in PBS and incubated at 17°C overnight. The anti-IHNV antibody had been prepared by preadsorbing the sera onto EPC cells for 1 h. Wells were subsequently washed with PBS and a biotinylated secondary antibody, goat anti-rabbit polyclonal (Promega), was added at a 1/1000 dilution in PBS and incubated at room temperature with gentle shaking for 30 min. Wells were rinsed again in PBS after which phosphatase-conjugated avidin-biotin complex (ABC) (Vectastain-ABC mouse IgG kit, Vector

Laboratories, Burlingame, CA) was added and the wells were incubated for another hour. After a final rinse with PBS, fixed cells were equilibrated by the addition of 100 mM Tris, pH 8.2 for approximately 3 minutes and Vector-Red phosphate substrate (Vector) in 100 mM Tris, pH 8.2 was added to the wells. Plates were placed in the dark to develop and reactions were terminated by the addition of distilled water.

DNA injections in rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) from the Oak Springs hatchery, Oregon were held at Oregon State University Fish Disease Laboratory in 12°C fish-pathogen-free water where subsequent DNA injections were carried out. The previously described plasmids, PCR-M1 and PCR-M2770 were used in DNA injection studies as follows. DNA concentrations were adjusted to 0.1 ug/ul by diluting in PBS, pH 7.4 . Four groups of 156 fish (1 g mean wt) per group were anesthetized in 50 ppm 3-aminobenzoic acid ethyl ester (Benzocaine, Sigma) and subsequently injected with either 100 uls of PBS as a negative control or 100 ul (10 ug) of each plasmid construct. Blood was collected from anesthetized fish at 2 week intervals for 6 weeks by severing the caudal peduncle and collecting the blood in heparanized 100 ul capillary tubes. For each group, triplicate samples which contained the blood of five fish were taken. Samples were stored on ice and the serum was collected by centrifugation at 14000g in a cold high speed centrifuge and stored at -70°C.

Immunoblotting

The specificity of the rainbow trout antisera was tested by immunoblotting. Purified IHNV (20 ug/lane) and the proteins in IHNV-infected CHSE-214 cell lysates were separated by PAGE and transferred onto immobilon-PSQ membranes (Millipore) at 50V for 45 minutes. Membranes were blocked in 5% non-fat milk, 1% BSA in TBS

overnight at 4°C. Membranes were then placed in a Mini-proteanII-multi-screen blotting apparatus (Bio-Rad). Trout serum samples 1/50 and 1/100 in TBS with 0.01% Tween 20 were applied to the wells (600 ul/well) and incubated overnight at 17°C. Membranes were removed from the apparatus and the antigenic proteins were detected with a phosphatase-labeled goat anti-trout IgG (Kirkegaard and Perry Laboratories, Inc.) as the secondary antibody. Membranes were rinsed in TBS with 0.01% Tween-20 and 1% BSA. Blots were developed with the BCIP/NBT phosphatase substrate for Westerns (Kirkegaard and Perry Laboratories, Inc). Trout sera from an IHNV-infected fish was used as a positive control.

Enzyme linked immuno-sorbant assays (ELISA)

The following ELISA protocol was used to detect IHNV antibodies in fish serum following DNA injection. Purified IHNV (3ug/ml) in a sodium carbonate buffer (15 mM Na_2CO_3, 5 mM $NaHCO_3$, NaN_3, pH 9.2) was coated onto the wells of a 96 well microtiter plate (Corning, EIA) overnight at 4°C. The wells were washed twice with Tris-buffered saline (TBS, 20mM Tris base, 500 mM NaCl, pH 7.5) containing 0.05% Tween-20 (TTBS) before adding a blocking solution of TBS containing 3% bovine serum albumin (BSA) (Sigma) for overnight at 4 °C. The wells were subsequently washed 3 times with TTBS after which two fold dilutions of collected rainbow trout sera (1/20) in a solution of TTBS containing 1% BSA (TTBSA) were added to the wells for overnight at 17°C. Wells were washed with TBTT after which phosphatase-labeled, goat anti-trout Ig 1/100 (Kirkegaard and Perry Laboratories, Inc.) was added for 1 hour at 37°C. Plates were washed 5 times with TBTT before the addition of a phosphatase substrate for ELISA (Kirkegaard and Perry Laboratories, Inc.) as the developer reagent. Plates were measured at an optical density of 405 nm using a Titertek Multiscan Plus plate reader (Flow Laboratories).

Results and Discussion

In vitro transcription and translation of plasmid DNA

To ensure that the plasmids, pCR3M1 and pCR3M2-770, did not contain gross errors introduced by PCR amplification *in vitro* translation reactions were carried out with the RNA transcribed from these plasmids. RNA synthesized *in vitro* was translated in a rabbit reticulocyte system in the presence of [^{35}S] methionine. Labeled, *in vitro* synthesized proteins are shown in Figure 4.1. The *in vitro* transcribed M1, (lane 1) and M2 (lane 2) comigrated with labeled M1(25 kd) and M2 (21 kd) from the infected cell lysates.(lane 4) In SDS-PAGE gels, M1 and M2 typically migrate at molecular weights which are higher than the predicted molecular weights. The band corresponding to the M2 protein is fainter than that of the M1. This may be due to several factors such as levels of RNA in translation reaction and efficiency of translation. The identity of the M1 translation product was confirmed by immunoblot analysis (Figure 4.2). *In vitro*- synthesized M2 could not be detected by immunoblotting. This is not suprising since the level of *in vitro* -synthesized protein is considerably lower than that for M1. These preliminary studies indicated that *in vitro*, pCR3M1 is a functional clone which encoded the M1 protein. The clones pCR3-M1 and pCR3-M2 that were used for the *in vitro* transcription/translation reactions were re-sequenced to confirm the plasmid constructions.

Humoral response following DNA injection

We were unable to detect anti-IHNV antibodies in trout sera collected 2, 4 and 6 weeks post DNA injection by immunoblotting or ELISA. It is uncertain why measurable antibodies were not detectable; however Anderson et al. (1995b) reported

Figure 4.1 Autoradiograph of *in vitro* translation products from pCR3-M1 and pCR3-M2. RNA was transcribed *in vitro* from plasmids pCR3-M1 and pCR3-M2770 and translated using a rabbit reticulocyte system using [^{35}S] as a radioactive label. Products were concentrated with a Centricon #3 filter and electrophoresed on a 12.5 % PAGE gel. Lane 1, pCR3-M1 translation product , lane 2, pCR3-M2770 translation product, lane 3, blank, lane 4, [^{35} S] labeled IHNV infected CHSE-214 cell extracts, and lane 5, labeled CHSE-214 cell extracts. Molecular weights for IHNV is: G, 67 kd; N, 42 kd; M1, 25 kd; M2, 21 kd.

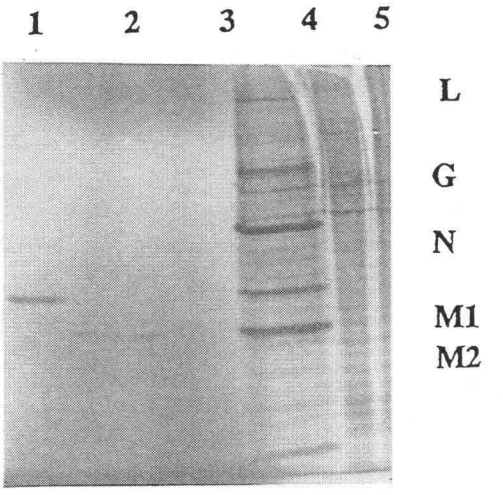

Figure 4.1

Figure 4.2 Immunoblot analysis of *in vitro* transcription/ translation products. Lane 1, M1, lane 2, M2 , lane 3, blank, lane 4, IHNV infected cell lysate, lane 5, uninfected cell lysate. Membranes were probed with anti-IHNV antibody.

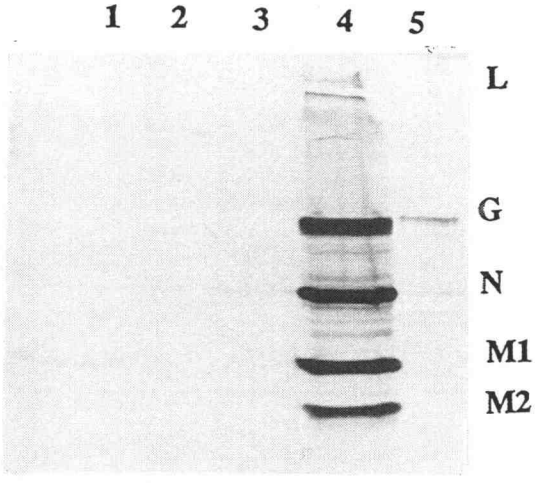

Figure 4.2

negative results for fish injected with plasmids encoding the nucleocapsid (N) protein of IHNV.

These results can not be explained by the inability of these proteins to elicit an antibody response in trout, since M1- and M2-specific antibodies are detected in trout sera following a natural IHNV infection (Ristow et al. 1993). Also, trout vaccinated with IHNV in Freund's complete adjuvant responded to G and M1 in immunoblots (Mourich and Leong 1991).

Other studies in which the endogenously produced internal viral proteins elicited only a weak or undetectable antibody response have also been reported (Larson et al. 1992, Fynan et al. 1993, Lawrence et al. 1988, Yokoyama et al. 1995 and Martins et al. 1995). In a study by Larson and co-workers, the phosphoprotein (M1) of rabies expressed from a vaccinia recombinant virus was unable to induce an measurable antibody response in mice; however the vaccinia-M1 did elicit a weak T-cell helper response and a strong T-cell cytotoxic response (Larson et al. 1992). In another study, low to undetectable titers in chicken sera of influenza hemagglutinin (HA) antibodies following DNA injection with an HA-expressing DNA was reported (Fynan et al. 1993). In this same study, a subsequent virus challenge resulted in levels of HA antibody which were much higher than what would be expected following a natural infection. This result suggests that the HA-expressed from DNA primed an HA-specific antibody response. This has also been reported for the N protein of IHNV where upon virus challenge N antibody levels were enhanced (Anderson et al. 1995). Yokoyama and coworkers (1995) reported very low levels of antiviral antibodies in lymphocytic choriomenigitis virus injection studies with the internal N protein and suggested that (1) proteins may be expressed at a low level because the mRNA are being expressed in a nuclear environment and perhaps don't follow their normal transcriptional and posttranscriptional pathways (2) proteins may not gain access to the appropriate antigen pathways if they remain inside the cell where they are not

presented to B cells or antigen-presenting cells. This may also be the case for M1 and M2 of IHNV.

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CHAPTER 5
SUMMARY

The complete nucleic acid and deduced amino acid sequences of the M1 phosphoprotein and M2 matrix protein genes of infectious hematopoietic necrosis virus (IHNV) were determined. The predicted M1 open reading frame (ORF) encodes a 230 amino acid protein with a estimated molecular weight of 25.6 kDa. Five possible phosphorylation sites were identified with the consensus sequence SXXE/D. In addition, a second ORF located within the M1 was identified which is 146 nucleotides in length with the potential to encode a 42 amino acid protein with a calculated weight of 4.8 kDa. This protein is highly basic and is similar to the small proteins found encoded within the phosphoprotein gene of vesicular stomatitis virus and rabies virus. The predicted M2 ORF is 739 nucleotides in length and encodes an 195 amino acid protein with a calculated molecular weight of approximately 22 kDa. The amino acid sequence of IHNV M1 and M2 were aligned with other rhabdoviruses to determine the extent of relatedness . IHNV is more closely related to the fish *Lyssaviruses*, hirame rhabdovirus and viral hemorrhagic septicemia than to the other rhabdoviruses.

Plasmids encoding the M1 or M2 matrix protein genes of the fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV) under the control of a viral promoter/enhancer were tested for their ability to elicit an antibody response in rainbow trout (*Oncorhynchus mykiss*) after injection of the DNA into skeletal muscle. The ability of each plasmid to produce antigenically accurate M1 and M2 proteins was initially verified by transcription/ translation reactions *in vitro*. The injected fish were bled at 2, 4 and 6 weeks postinjection, and the production of antibody to M1 and M2 in rainbow trout was monitored by immunoblotting. IHNV-specific antibody titers in fish sera were monitored over a 6 week period by enzyme-linked immunosorbant assay (ELISA). No antibody was detected within that period. However, this was not unexpected since these

proteins are not transferred to the cell surface. This work may prove useful in the development of an effective DNA-based subunit vaccine against IHNV.

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